

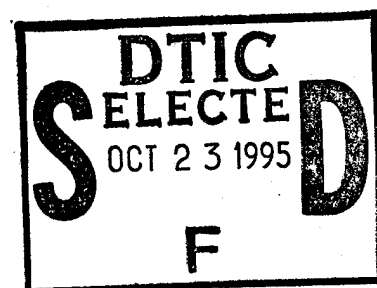
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During Development and in Breast Cancer

PRINCIPAL INVESTIGATOR: Linda M. Varela  
Margot M. Ip, PhD

CONTRACTING ORGANIZATION: Health Research, Inc.  
Buffalo, New York 14263



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Linda M. Varela 7/25/95  
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## 1. INTRODUCTION

**I. Nature of problem:** The risk that a woman in the U.S. will develop breast cancer in her lifetime has now increased to an unprecedented one out of eight women, and breast cancer is currently the most common cancer among women. It is therefore crucial that a better understanding of those factors leading to the development of breast cancer and of the metastatic phenotype be achieved so that more appropriate strategies for its prevention and/or treatment can be applied. This can be achieved by a thorough investigation of the normal mammary gland, and through a subsequent comparison of this with malignant breast cells, important clues as to how breast cancer evolves may be discovered. While the fundamental causes for breast cancer remain elusive, a growing body of data suggests that the major risk factors may be inherently biological, such as natural hormone or growth factor production (1). Thus, by examining the cyclical variations in hormone levels, the complex hormonal regulation of proliferation and differentiation, and the changes in gene expression that occur as the mammary gland progresses through the different stages of development, the prospects for prediction, prevention, and treatment of breast cancer may be heightened. A possible key player in this intricate network is tumor necrosis factor-alpha ( $\text{TNF}\alpha$ ).  $\text{TNF}\alpha$  has been shown by our laboratory to play a significant role in directing both the proliferation as well as the morphological and functional differentiation of mammary epithelial cells (2). This regulator may reach the MEC not only via traditional endocrine and local paracrine routes, but there may also be autocrine synthesis of  $\text{TNF}\alpha$  by the RMEC as well. Normally, there is strict control of the expression of this cytokine; however, it is possible that any disruption of this control has the potential to markedly affect the degree of both growth and differentiation and may confer on the cell a transformed phenotype. Thus, it is critically important to determine the physiological role of  $\text{TNF}\alpha$  in the growth and development of the mammary gland and how this role differs in transformed cells, so that more appropriate strategies for the prediction, prevention, and treatment of breast cancer may be developed.

**TUMOR NECROSIS FACTOR  $\alpha$  ( $\text{TNF}\alpha$ ):**  $\text{TNF}\alpha$ , a 17-kDa pluripotent protein, was originally defined by its antitumor activity both *in vitro* and *in vivo* (3). It is now known, however, that  $\text{TNF}\alpha$  mediates a diverse array of cellular responses, such as regulation of its own production, cytotoxicity, and inflammation, by acting alone or in concert with a variety of other cytokines, hormones or growth factors (4-6).  $\text{TNF}\alpha$  also regulates the growth, differentiation, and function of virtually all cell types, including normal rat mammary epithelial cells in culture (2,7-9). In addition,  $\text{TNF}\alpha$  has been implicated as the causative agent in the hemorrhagic necrosis of tumors; however, the excessive levels of  $\text{TNF}\alpha$  necessary to effect this response are also associated with the detrimental phenomena of septic shock and cachexia, which, along with resistance, are the primary factors responsible for the failure of  $\text{TNF}\alpha$  as a cancer chemotherapeutic agent (10-13). This complex physiology may be the result of different forms of  $\text{TNF}\alpha$ : the 17-kDa secretory form, the 26kDa transmembrane precursor form, which is proteolytically cleaved to form the mature 17-kDa  $\text{TNF}\alpha$ , or both. The 17-kDa form may be responsible for systemic effects such as septic shock or cachexia due to widespread

release into the circulation, while the transmembrane form may act locally via cell-to-cell interactions (14). In addition, this diversity may also be due to complex regulation of  $\text{TNF}\alpha$  expression.  $\text{TNF}\alpha$  synthesis is controlled by numerous variables, including hormones, cytokines, phorbol esters, and bacterial toxins, and  $\text{TNF}\alpha$  expression is also stringently regulated on a post-transcriptional level (15-19). Lastly, the pleiotropic effects of  $\text{TNF}\alpha$  may be mediated through different receptors and alternate signal transduction pathways.

While these parameters have been characterized in cell types such as fibroblasts and various immune cells, there is currently no information available on the capability of mammary epithelial cells to produce  $\text{TNF}\alpha$ , and no information on its physiological role, if any, in directing the overall growth and development of the mammary gland.

**TNF $\alpha$  RECEPTORS:** The multiple biological activities of  $\text{TNF}\alpha$  are mediated through a family of cell surface receptor proteins. Two immunologically distinct  $\text{TNF}\alpha$  binding proteins of 55- and 75-kDa have been identified (20-23). In addition, soluble forms of the two receptors also exist, corresponding to the extracellular domains of the transmembrane proteins. These soluble receptors serve as natural inhibitors of  $\text{TNF}\alpha$  and may provide a regulatory mechanism for the modulation of excess  $\text{TNF}\alpha$  released during injury or infection (21,24-29). The two major receptor forms have limited sequence homology in their extracellular domains, and no significant homology has been found between their intracellular regions. This suggests that the two receptor types may activate different signal transduction pathways, which would further contribute to the diversity of  $\text{TNF}\alpha$ 's actions (30). In order to determine the individual roles of the two  $\text{TNF}\alpha$  receptors, both agonistic and neutralizing antibodies directed against the two receptors have been developed. Interestingly, studies employing these tools have demonstrated that signals from these receptors are not redundant, but rather are distinct. For example, antibodies to the 55-kDa receptor have been shown to initiate the signal for cellular cytotoxicity, while the 75-kDa receptor has been shown to initiate the proliferation of certain cell types (22,31,32).

Since  $\text{TNF}\alpha$  is able to regulate both the growth and differentiation of normal rat mammary epithelial cells in culture, it is hypothesized that one or both  $\text{TNF}\alpha$  receptors are present on the RMEC; however, as with the overall production of  $\text{TNF}\alpha$ , there is currently no information on  $\text{TNF}\alpha$  receptor expression in normal mammary epithelial cells. Consequently, the functional roles of these receptors have not been identified. Thus, characterization of both receptor expression and functionality may lead to the complementation of existing breast cancer therapies or to the development of new treatments altogether.

**II. Previous studies:** Our laboratory has developed a unique model system for the primary culture of rat mammary epithelial cells (RMEC). In this model, cells are cultured within an EHS-derived reconstituted basement membrane matrix in a defined serum-free medium containing all the factors necessary for optimal growth and differentiation, including prolactin, EGF, progesterone, insulin, and hydrocortisone. In

this system, undifferentiated RMEC differentiate, both morphologically and functionally, to an extent comparable to that of the lactating mammary gland, and the hormonal and growth factor regulation of differentiation in this model correlates with *in vivo* development. The RMEC also proliferate extensively, and this can readily be quantitated (33-35). Using this model system, our laboratory has shown that  $\text{TNF}\alpha$  is also able to regulate both the growth and development of RMEC in culture. Specifically,  $\text{TNF}\alpha$  was shown to stimulate RMEC proliferation under both optimal media conditions as well as in medium deficient in EGF.  $\text{TNF}\alpha$  had no effect on morphological differentiation in optimal conditions, but had a marked stimulatory effect in medium either lacking or deficient in EGF. The effect of  $\text{TNF}\alpha$  on functional differentiation was more complex, with the results suggesting that  $\text{TNF}\alpha$  may have a direct inhibitory effect on casein gene expression and thus functional differentiation (2). This was the first study to report that  $\text{TNF}\alpha$  is a potential regulator of mammary gland development, so further investigation of this role is critical.

In summary, tumor necrosis factor  $\alpha$  has been shown to exert a marked effect on the proliferation as well as morphological and functional differentiation of mammary epithelial cells. These effects are presumed to be mediated via an interaction of  $\text{TNF}\alpha$  with specific cellular receptors. A large number of physiological regulators modulate the synthesis, secretion, and function of  $\text{TNF}\alpha$  in various cell types. Additionally, the expression and function of  $\text{TNF}\alpha$  may be altered in some malignant cells, including breast cancer cells. Since loss or alteration of the  $\text{TNF}\alpha$  signaling pathway could markedly affect cellular proliferation and differentiation, the altered cell may no longer be controlled by normal environmental restraints and become highly proliferative as well as invasive. Currently, the role of  $\text{TNF}\alpha$  in directing the cellular development of mammary gland is, at best, poorly understood. To date, no work has been published concerning the capability of normal mammary epithelial cells to produce  $\text{TNF}\alpha$ , or directly comparing  $\text{TNF}\alpha$  effects in normal and transformed MEC. It is therefore the objective of this grant to investigate the synthesis of  $\text{TNF}\alpha$  by normal MEC, its role and regulation during development, and how these parameters are altered in malignant MEC.

**III. Purpose of present work:** The growth and development of the mammary gland is under a myriad of regulatory controls, including endocrine hormones and growth factors which are important regulators of proliferation, differentiation, and ultimately involution during the various stages of development. In addition, local growth factors may be produced by the mammary epithelial cells (MEC) themselves or by the complex surrounding stromal matrix of the MEC.  $\text{TNF}\alpha$  may play a crucial role in this intricate network, since previous work in our laboratory demonstrated that  $\text{TNF}\alpha$  was able to regulate the growth as well as the morphological and functional differentiation of normal MEC.



Several questions arose from these preliminary observations, the foremost being what is the endogenous source of the  $\text{TNF}\alpha$  that mediates these effects *in vivo*? It is already known that cells such as fibroblasts and lymphoid cells, located elsewhere in the body but which are also found in the complex stromal matrix of the mammary gland, can produce  $\text{TNF}\alpha$ . In addition, some human breast cancer cells are able to produce  $\text{TNF}\alpha$ , so it is possible that normal breast cells may also express this cytokine (16,36-41). Also,  $\text{TNF}\alpha$  is cytotoxic to many cancer cells, including breast cancer cells (7,42,43); however,  $\text{TNF}\alpha$  has been shown to stimulate the proliferation of various other cell types, including normal RMEC (2,7).  $\text{TNF}\alpha$  is also able to alter the morphology of both normal and malignant breast epithelial cells. Unfortunately, there is currently no information concerning the capability of normal mammary epithelial cells to produce  $\text{TNF}\alpha$  and the *in vivo* role of  $\text{TNF}\alpha$  in directing mammary gland development is, at best, poorly understood. Thus, in order to better understand the role of  $\text{TNF}\alpha$  in the regulation of MEC proliferation and differentiation, the overall objective of the proposed studies is to determine whether  $\text{TNF}\alpha$  is a physiologically relevant regulator of mammary gland growth and development.

Since  $\text{TNF}\alpha$  stimulates proliferation and morphological differentiation and inhibits casein production in normal RMEC, it may play a role in directing the proliferation and branching of the MEC into the fat pad during puberty and in alveolar development in early pregnancy (during which time casein synthesis is inhibited) (44). This function has obvious implications in the development of metastasis, for if the controlled invasion directed by  $\text{TNF}\alpha$  were deregulated or disrupted, it could potentially lead to uncontrolled invasion and the development of metastatic cancer. In support of this theory, our laboratory has found that  $\text{TNF}\alpha$  induces metalloproteinase (92 kDa gelatinase B) activity.  $\text{TNF}\alpha$  could also participate in the involution of the mammary gland at the end of lactation, perhaps via the induction of apoptosis (as has been observed in response to  $\text{TNF}\alpha$  in other tissues) (5,45). Thus,  $\text{TNF}\alpha$  may be growth stimulatory during mammary gland development but cytotoxic after lactation, which could perhaps be due to the presence of an inhibitor of cytotoxic activity during development (46,47). Thus, *in vivo* studies in rats during various stages of development will allow assessment of  $\text{TNF}\alpha$  levels and possibly cytotoxic activity at various stages in the hopes of identifying potential roles of  $\text{TNF}\alpha$ , and through a correlation with documented literature values of *in vivo* hormone levels at these various stages, an understanding of the *in vivo* regulatory control of  $\text{TNF}\alpha$  production in the mammary gland may be achieved.

Since  $\text{TNF}\alpha$  is able to regulate the growth and differentiation of normal RMEC, then one or both  $\text{TNF}\alpha$  receptors should theoretically be present on the RMEC; however, the specific  $\text{TNF}\alpha$  receptors present on RMEC have not yet been identified. If they are not, then it must be determined what alternate receptors  $\text{TNF}\alpha$  is able to act through. Receptor expression may also be developmentally or hormonally controlled, and this potential variation in expression may contribute to the myriad of effects  $\text{TNF}\alpha$  is able to initiate and may also be associated with progression to malignancy.

In addition to determining receptor expression, it is critical to determine receptor functionality. The functional roles of the two major receptor forms are still the subject of much controversy, and these roles appear to vary among species. In many cases, the 55-kDa receptor mediates the signal for cellular cytotoxicity, and while the functional roles of the 75-kDa receptor are largely unknown, it does appear that proliferation in some systems is signaled through this receptor (22,31,32,48). Thus, it is possible that the differential effects of  $\text{TNF}\alpha$  on the RMEC (stimulation of proliferation and morphological differentiation and inhibition of functional differentiation) may be mediated through the two different receptors and/or alternate signal transduction pathways. Stimulation of proliferation may be mediated via one particular receptor, while inhibition of functional differentiation or cytotoxicity may be initiated through another receptor. Currently, there is no information available concerning the functionality of  $\text{TNF}\alpha$  receptors on RMEC. If the receptor proteins and their respective functional significance can be identified, it may be possible to develop new, improved therapies for breast cancer, or to complement existing therapies in order to enhance their effectiveness. Stimulation of a specific receptor subtype could potentially be used to initiate a cytotoxic response, while neutralizing the signalling of another receptor could be used to inhibit a proliferative response.

Thus, the overall goal of these studies is to determine the regulation and role of  $\text{TNF}\alpha$  during the various stages of mammary gland development and in malignancy, with the hope of gaining insight into the design of more rational breast cancer therapies.

#### **IV. Methods of approach:**

A. *Investigation of the hormonal regulation of  $\text{TNF}\alpha$  mRNA and protein expression by normal RMEC.* This focus of this aim was the detection of  $\text{TNF}\alpha$  mRNA and protein production as well as its hormonal regulation. The effect of removing various hormones from the optimal serum-free culture medium on  $\text{TNF}\alpha$  production was to be assessed. In conjunction with the *in vitro* studies, *in vivo* studies of  $\text{TNF}\alpha$  expression in the mammary glands of virgin, pregnant, lactating, and involuting rats were performed.

B. *Identification of the specific TNF receptors present on normal RMEC.* This aim focused on the identification of TNF receptors present on normal RMEC and attempted to identify which particular cellular effects were mediated by each of the receptors.

Techniques to be used in these studies included primary culture, preparation and utilization of reconstituted basement membrane matrix, RMEC isolation at various developmental stages, light microscopic analysis, Northern blot and Western blot analysis, RNA and protein electrophoresis, DNA synthesis, RNA and protein determination, bioactivity assay, and statistical analysis. Agents to be used in these studies included murine  $\text{TNF}\alpha$  and TNF receptor cDNAs, agonistic and neutralizing antibodies, and antibodies for Western blot analysis.

## 2. BODY

### (A) Hormonal regulation of TNF $\alpha$ expression

**(i) *In vitro* studies** All studies in this aim were conducted using freshly isolated RMEC in primary culture, with cells seeded within an EHS-derived reconstituted basement membrane matrix and cultured with a defined serum-free medium.

In the first set of studies, TNF $\alpha$  mRNA expression by normal RMEC in culture was examined and correlated with the level of morphological differentiation. The hormonal regulation of TNF $\alpha$  production by RMEC in culture was assessed by removing one hormone at a time (prolactin, progesterone, hydrocortisone, or EGF) from the optimal culture medium and then assessing mRNA expression. Estrogen, at a physiological concentration of  $10^{-9}$  M, was also added into the culture medium for various times prior to analysis.

At various stages of *in vitro* differentiation, RNA was isolated from the RMEC using a guanidinium isothiocyanate/phenol-chloroform procedure. The RNA was then analyzed via Northern blot analysis using a mouse TNF $\alpha$  oligonucleotide probe that is known to cross react with rat. Both the oligonucleotide probe and experimental procedure were generous gifts from a colleague at the University of Buffalo. Morphological differentiation was assessed by counting the different colony types at various times throughout the 21 day culture period. Typically the following colony types are quantitated: immature, end bud-like, lobular, multilobular, simple ductal, multilobular-ductal, hybrids of end bud-like and lobular or multilobular, and squamous-like (35).

Unfortunately, it was discovered that the oligonucleotide probe used in the Northern blot procedure was not identifying TNF $\alpha$ , but rather was binding non-specifically to the 28S RNA band. Thus, the results we thought we had obtained were not valid. Extensive time and effort was then put into modifying the procedure for Northern blot analysis of TNF $\alpha$ , and the following changes were made: a cDNA probe specific for rat TNF $\alpha$  was obtained from another investigator, and after testing numerous methods, the optimal hybridization conditions for its use were determined using poly A+ mRNA (it was also discovered that TNF $\alpha$  RNA levels were too low to be detectable with total RNA). Results using this revised method allow us to state with certainty that TNF $\alpha$  is expressed by the epithelial cells of the mammary gland. No further results on the *in vitro* hormonal regulation of TNF $\alpha$  expression have been obtained; however, in order to obtain more physiologically relevant information for future potential *in vitro* studies, we have decided to focus on the *in vivo* regulation of TNF $\alpha$  and its receptors.

In conjunction with the RNA studies,  $\text{TNF}\alpha$  protein levels were examined via Western immunoblot analysis using a polyclonal antibody against murine  $\text{TNF}\alpha$  that is cross-reactive with rat. (It was first necessary to test several anti-murine  $\text{TNF}\alpha$  antibodies for their cross-reactivity with rat because no anti-rat specific antibodies are available.) After optimization of conditions, RMEC plus matrix, cells digested free of the matrix, or matrix cultured in the absence of cells (control) were lysed and the supernatant subjected to SDS-PAGE and immunoblot analysis. Protein secretion was examined by subjecting media samples to SDS-PAGE and immunoblot analysis. Thus far, we have failed to detect any  $\text{TNF}\alpha$  protein product in the media of normal RMEC. Importantly, however, preliminary immunoblot analysis of cell lysates indicates the presence of a faint 26 kDa band which may represent the membrane-bound, precursor form of  $\text{TNF}\alpha$ .

**(i) *In vivo* studies** Coupled with the *in vitro* studies discussed above, *in vivo* studies of  $\text{TNF}\alpha$  production in the mammary glands of virgin, mid-pregnant, lactating, and post-lactating rats are currently being performed. Both the MEC and stromal components from the aforementioned rats are being examined for  $\text{TNF}\alpha$  mRNA and protein production, and for bioactivity.  $\text{TNF}\alpha$  mRNA and protein production are being analyzed using the Northern and Western blot methods described above. Bioactive  $\text{TNF}\alpha$  production is being quantitated using a standard WEHI biological assay for cytotoxic activity. Extracts of RMEC isolated from the mammary glands of rats during the various stages of development mentioned above are being tested for bioactivity; however, numerous technical problems are being encountered in the bioactivity assay, so the proper conditions are still being sought.

The isolation of the epithelial cells from the rats at the various reproductive stages follows the same basic procedure as is used for the virgin gland; however, a significant amount of time was necessary in order to optimize both the digestion time and conditions for the mammary glands from these different stages. Not only do the glands themselves have a different composition and consistency than the virgin gland, but the RMEC are much more fragile and difficult to work with. Now that these details are completely worked out, we are proceeding to finish these experiments. We also hope to assess the levels of  $\text{TNF}\alpha$  and its two receptors in mammary stromal cells, although conditions for their isolation still have to be worked out. Moreover, the ratios of these various cell types change greatly during the different developmental stages, making it difficult to obtain sufficient quantities of these cells for use.

**(B) Identification of  $\text{TNF}\alpha$  receptors on normal RMEC** Because the presence of  $\text{TNF}$  receptor mRNA on RMEC has not previously been reported, we wanted to determine receptor mRNA expression and regulation on RMEC both *in vitro* and *in vivo*. Again, after extensive optimization of the hybridization procedure, the proper conditions were determined using poly A<sup>+</sup> mRNA and cDNA probes specific to either the 55- or 75-kDa  $\text{TNF}$  receptors. Unfortunately, no data on the *in vitro* expression of either  $\text{TNF}$  receptor mRNA species is available because all samples were used in the first series of  $\text{TNF}\alpha$  Northern blots that failed. However, figure 1 shows that poly A<sup>+</sup> mRNA from freshly isolated virgin RMEC *in vivo* contain both p55 and p75  $\text{TNF}$  receptor species. A

single mRNA species of 2.3 kB was identified for the p55 TNF receptor, which is consistent with previous reports in other tissues (25,49,50). No additional bands were detected. In contrast, Northern blot analysis revealed three transcripts of 5.7, 4.2 and 2.8 kB for the p75 TNF receptor in virgin RMEC. The 4.2 and 2.8 kB species predominated, while the 5.7 kB mRNA was relatively minor. Studies of TNF receptor mRNA expression *in vivo* on RMEC isolated from the mammary glands of pregnant, lactating, and post-lactational rats are currently underway; however, no results are available at this time.

In addition to the mRNA studies, antibodies specific to either the 55- or 75-kDa receptor will be used to confirm which receptor subtypes are present on the RMEC, both *in vitro* and *in vivo*. Thus far, two major limitations have been encountered prior to undertaking these studies. First, obtaining the appropriate rat-reactive antibodies has been an obstacle. As there are no rat-specific antibodies available, we have attempted to obtain anti-murine species in the hopes that they would cross-react with rat. Unfortunately, very few are available (most are anti-human and they either do not cross-react with mouse/rat or the cross-reactivity is unknown), and of those that do exist, we cannot obtain a sufficient quantity necessary to perform the experiments. In conjunction, a second problem is that TNF receptor protein levels are extremely low, so large amounts of protein must be available for some form of purification in order to facilitate detection.

**Functional assessment of TNF $\alpha$  receptors *in vitro*** Because previous studies in our laboratory demonstrated that TNF $\alpha$  was able to affect both the growth and differentiation of RMEC in primary culture, we wanted to determine which TNF receptor was mediating which specific TNF $\alpha$  effect. In order to assign specific functions to the individual receptors, RMEC in primary culture were incubated with agonistic antibodies highly specific to either the p55 or p75 receptors (51). These antibodies were generous gifts of Genentech; however, we were only able to obtain a very limited quantity of each of the antibodies. Our studies thus focused primarily on the ability of the anti-TNF receptor antibodies to mimic either the stimulation of proliferation by TNF $\alpha$ , or the alteration of both morphological and functional differentiation.

In the first set of studies, the effect of the anti-receptor antibodies on RMEC proliferation was assessed by a [ $^3$ H]-thymidine incorporation assay in which RMEC were cultured until day 5 in suboptimal serum-free medium containing the optimal concentration of all components except EGF, which was decreased from 10 to 0.1 ng/ml. (This particular medium condition was used because TNF $\alpha$  itself was able to stimulate RMEC proliferation in this reduced EGF state.) The cells were then incubated for 48 hours in the presence of either EGF (10ng/ml), TNF $\alpha$  (40 ng/ml), or various dilutions of the receptor-specific agonistic antibodies. The RMEC were then pulse-labeled with [ $^3$ H]-thymidine for the last 4 hours of their 48 hour treatment. The acid-insoluble fraction of the RMEC was precipitated and [ $^3$ H]-thymidine incorporation was determined by liquid scintillation counting. Figure 2 shows that EGF, TNF $\alpha$ , and the 1:1000 dilution of the p55 antibody were able to significantly stimulate thymidine

incorporation by RMEC (~3 fold), while none of the tested dilutions of the p75 antibody had any effect on RMEC proliferation.

For morphological analysis, the RMEC were cultured in conditions identical to those used for the [ $^3$ H]-thymidine assay. The morphological development of the mammary epithelial organoids was quantitated during the last 4-6 hours of their 48 hour treatment via light microscopic observation. Two main colony types were classified, end bud and alveolar, with subcategories in each to distinguish between simple, lobular, and lobulo-ductal organoids. End bud colonies were defined by their simple, lobular structure and "rust" colored appearance, while alveolar organoids were defined by their more complex lobular structure, dark gray or black color, and the presence of lipid droplets within the structure. Figure 3 shows the gross morphological appearance of the organoids after treatment. Organoids cultured in the 0.1 ng/ml EGF control medium were end bud in appearance, defined by their "simple" lobular structure and "rust" color, while treatment with either EGF,  $\text{TNF}\alpha$ , or the 1:1000 dilution of either the p55 or p75 antibody was able to shift the morphology of the organoids toward the more differentiated lobulo-alveolar phenotype. The figure also shows that treatment significantly increased both the number and extent of ductal projections emanating from the colonies. Graphical quantitations of the effect of the TNF receptor antibodies on overall end bud and alveolar colony morphology are shown in figures 4 and 5. The graph in figure 4 shows that in the 0.1 ng/ml EGF control, the colonies are primarily (~85%) of the undifferentiated end bud type, while in EGF- or  $\text{TNF}\alpha$ - treated groups, the percentage of end bud-like colonies significantly decreased to ~20-25% of the total colonies. Interestingly, both TNF receptor agonistic antibodies were able to affect the morphology in a concentration-dependent manner, with the 1:1000 dilution of each having the greatest effect. In contrast, figure 5 shows that in the 0.1 ng/ml EGF control, the colonies are only ~10% alveolar, while in EGF- or  $\text{TNF}\alpha$ - treated groups, the percentage of differentiated alveolar-like colonies significantly increased to ~75-80% of the total colonies. Again, both TNF receptor agonistic antibodies were able to stimulate the morphological development of the RMEC in a concentration-dependent manner.

The effect of the TNF receptor agonistic antibodies on the functional differentiation of primary RMEC was then assessed by measurement of casein accumulation by Western blot analysis. In the first series of Western blotting experiments, the RMEC were again cultured in conditions identical to those used for both the [ $^3$ H]-thymidine assay and the morphology studies (ie: suboptimal EGF). The RMEC were harvested and the supernatant (adjusted to equivalent protein content by Biorad analysis) was subjected to electrophoresis and Western blotting with rabbit polyclonal antiserum against the rat casein proteins. The Western blot in figure 6 shows that EGF significantly increases the accumulation of all 4 forms of casein ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$ ) by RMEC, while  $\text{TNF}\alpha$  had the opposite effect, with primarily the  $\alpha_2$ ,  $\beta$ , and  $\gamma$  forms being decreased. This figure also shows that the TNF receptor agonistic antibodies had opposing effects; RMEC treated with the 1:1000 dilution of the p55 receptor antibody showed a decreased accumulation of  $\alpha_2$ ,  $\beta$ , and  $\gamma$  casein, but cells treated with the 1:10<sup>4</sup> and 1:10<sup>5</sup> dilution of the p75 antibody showed an increase in accumulation of primarily  $\beta$  and  $\gamma$  caseins.

For a second series of Westerns, RMEC were cultured until day 5 in optimal, serum-free medium (containing 10ng/ml EGF) in order to better assess any decreases in casein accumulation. The cells were then incubated for 48 hours in the presence of either TNF $\alpha$  (40 ng/ml) or various dilutions of the TNF receptor agonistic antibodies. The harvested RMEC extract was then subjected to electrophoresis and Western blotting as described above. The Western blot in figure 7 shows that TNF $\alpha$  significantly decreases the accumulation of all 4 forms of casein ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$ ) by RMEC, with the primary effect on  $\alpha_2$ ,  $\beta$ , and  $\gamma$ . The figure also shows that the TNF receptor agonistic antibodies again had opposing effects; RMEC treated with the 1:10<sup>3</sup> and 1:10<sup>4</sup> dilutions of the p55 receptor antibody showed a decreased accumulation of  $\alpha_2$ ,  $\beta$ , and  $\gamma$  casein, but cells treated with the 1:10<sup>-4</sup> and 1:10<sup>-5</sup> dilutions of the p75 antibody showed an increase in casein accumulation. Figure 8 depicts a graphical quantitation of these results.

In light of these unexpected, contrasting results, we decided to investigate the potential mechanisms whereby these receptors may be mediating their opposite effects. Several potential mechanisms could explain these opposing effects: the two receptors may mediate, independently, changes in the rate or level of gene transcription, altered message stability, changes in the rate or level of casein protein synthesis, or may cause degradation or secretion of the casein proteins. Thus, we decided to first determine what effect, if any, the two receptors had on casein synthesis. The RMEC were cultured in optimal (10 ng/ml) serum-free medium until day 5; the cells were then treated for 48 hours with various dilutions of either the p55 or p75 TNF receptor agonistic antibodies and labeled with [<sup>35</sup>S]-methionine for the last 24 hours of their incubation. The radiolabeled casein proteins were immunoprecipitated, electrophoresed, and subjected to fluorography, and the amounts of newly synthesized casein proteins were quantitated by densitometric analysis. The results of this experiment are shown in figure 9: the TNF receptor agonistic antibodies had opposing effects on casein *synthesis* by RMEC. Treatment with the p55 TNF receptor antibody caused a suppression of casein synthesis, with the greatest decreases occurring in  $\alpha_1$  and  $\gamma$ . In contrast, treatment with the p75 TNF receptor antibody stimulated synthesis of  $\alpha_1$  and  $\gamma$  caseins, while  $\alpha_2$  and  $\beta$  were unaffected. As this experiment was only performed once, it will have to be repeated in order to confirm these results.

### 3. CONCLUSIONS

In the present studies, we have attempted to determine both the *in vitro* and *in vivo* expression of both TNF $\alpha$  and its two receptors, and to assess the potential functional role of each of the receptors in the mammary gland. Unfortunately, numerous technical problems were encountered during the *in vitro* studies of both TNF $\alpha$  and the receptors; thus, no data on *in vitro* TNF $\alpha$  or receptor mRNA expression is available. Conditions have now been optimized, however, so results should be forthcoming in the near future.

Preliminary Western blots have thus far failed to detect any  $\text{TNF}\alpha$  protein in the medium of RMEC cultured *in vitro*; however, a faint 26 kDa band has been detected in cell lysates of RMEC which may represent the membrane-bound, precursor form of  $\text{TNF}\alpha$ . Several possible explanations may explain this failure to detect the 17 kDa soluble form of  $\text{TNF}\alpha$ : the level of secreted  $\text{TNF}\alpha$  may be below the limits of detection,  $\text{TNF}\alpha$  may be trapped in the lumen of the RMEC and thus not secreted into the medium bathing the basal surface of the cells,  $\text{TNF}\alpha$  may be secreted but trapped in the EHS-basement membrane, or soluble  $\text{TNF}\alpha$  receptors may be present which may bind the  $\text{TNF}\alpha$  and could thus prevent antibody recognition of  $\text{TNF}\alpha$ . The 26 kDa band may represent a nonsecretable, cell-surface bound form of  $\text{TNF}\alpha$  produced by the RMEC; this protein may still be biologically active but only able to exert its effect via cell-to-cell interactions. This possibility has been documented in other cells by the discovery of a bioactive, mutant, and uncleavable transmembrane form of another growth factor,  $\text{TGF}\alpha$  (14,52-54).

The studies of *in vivo*  $\text{TNF}\alpha$  mRNA and protein expression in RMEC isolated from the mammary glands of virgin, pregnant, lactating, and post-lactational rats are currently underway; however, the studies are still in progress, so there are no results to report at this time, except that the studies are progressing well. If isolation of the various stromal components for the *in vivo*  $\text{TNF}\alpha$  studies continues to remain a problem, both the pattern of expression and localization of  $\text{TNF}\alpha$  mRNA and protein in the mammary stroma may be achieved by *in situ* hybridization and immunohistochemistry, respectively. Currently, a new member of the laboratory is optimizing the conditions for these assays, so these experiments should be feasible in the near future.

As mentioned above, no data is available on the *in vitro* expression of the  $\text{TNF}$  receptors due to the technical problems encountered; however, freshly isolated RMEC from virgin rats were found to express mRNA for both the p55 and p75  $\text{TNF}$  receptors. A single mRNA species of 2.3 kB was detected for the p55 receptor, while three transcripts of 5.7, 4.2, and 2.8 kB were found for the p75 receptor. The finding of only one mRNA transcript for the p55 receptor is consistent with previous reports, while for the p75 receptor, numerous mRNA species have been reported, ranging from 3.0 to 5.0 kB, so this variability is not without precedent but is still of significant interest (25,30,49,50). Whether or not this will translate into functionally distinct proteins with different locations or functions remains to be seen. Studies of  $\text{TNF}$  receptor mRNA expression *in vivo* on RMEC isolated from the mammary glands of pregnant, lactating, and post-lactational rats are currently underway; however, no results are available at this time. In addition, future efforts will focus on the determination of  $\text{TNF}$  receptor mRNA localization and expression via *in situ* hybridization.

Unfortunately, both the *in vitro* and *in vivo* studies of the  $\text{TNF}$  receptor *proteins* have not been possible due to limitations on both the amounts of material needed for study and the availability of the proper antibodies. Currently, we are in communication with two different investigators who have privately produced several anti-p55 and anti-p75



TNF receptor antibodies in attempts to obtain samples for our use. Even though they are anti-human and anti-murine in nature, they may also show cross-reactivity in our rat system. If the appropriate antibodies are obtained, we would also like to determine TNF receptor protein expression and localization via immunohistochemistry.

The data on TNF receptor functionality demonstrate that in normal MEC, the functions of  $\text{TNF}\alpha$  are divided between the two distinct TNF receptors, p55 and p75. The p55 receptor was found to be the sole mediator of  $\text{TNF}\alpha$ -induced proliferation, while both receptors were able to transduce the stimulatory signal for morphological development. The effects on casein accumulation, however, were more complex, with inhibition occurring through the p55 receptor and stimulation through p75. Moreover, the inhibitory signal of the p55 receptor occurred at a higher concentration (lower dilution) than did the stimulation triggered by the p75 receptor ( $1:10^3$  versus  $1:10^4$  and  $1:10^5$ , respectively). When taken in conjunction with earlier data obtained by our laboratory in which  $\text{TNF}\alpha$  showed a biphasic effect on casein accumulation (in the absence of EGF), the data suggests that at low concentrations,  $\text{TNF}\alpha$  may primarily act via the p75 TNF receptor, while at higher concentrations  $\text{TNF}\alpha$  signalling may occur via p55. In addition, preliminary data herein suggests that these effects on casein are mediated, at least in part, via changes in the level of casein *synthesis*. When compared to the results on casein accumulation, the results agree in terms of overall effect; however, the effects on the various specific forms of casein are slightly different. The

$\alpha_2$ ,  $\beta$ , and  $\gamma$  caseins are more affected in terms of accumulation, while the synthesis of  $\alpha_1$  and  $\gamma$  show the greatest changes in response to treatment. This may be explained by the fact that accumulation reflects a balance among synthesis, degradation, and secretion, and these other factors must be examined in order to determine the relative role of each process. In the future, further investigation of the mechanism whereby  $\text{TNF}\alpha$  exerts its effects on casein may focus on the role of  $\text{TGF}\alpha$  and the EGF receptor. The p75 receptor has been shown to up-regulate  $\text{TGF}\alpha$  mRNA, whereas p55 transduces the signal for up-regulation EGF receptor mRNA (55). Since we have observed that the effect of  $\text{TNF}\alpha$  on casein requires 24-48 hours to occur, it is possible that  $\text{TNF}\alpha$  may affect casein indirectly through one or both of these factors in our system.

Thus, from the results obtained at this time, we hypothesize that during pregnancy when extensive proliferation and morphological development occur,  $\text{TNF}\alpha$  may act primarily through the p55 receptor. This particular role for the p55 TNF receptor may also have significant implications in the development of breast cancer, because it indicates that p55 could also be involved in stimulating tumor proliferation as well. During early pregnancy, casein gene expression is also inhibited, so this hypothesis correlates with our data in which the p55 receptor signals both a stimulatory effect on proliferation and an inhibitory effect on casein. During late pregnancy and lactation, however,  $\text{TNF}\alpha$  may shift its actions to the p75 receptor in order to "shut down" growth but continue alveolar development and "turn on" casein production.

**ABSTRACTS/POSTERS PRESENTED:**

1. Poster presented at American Association for Cancer Research Meeting in Toronto, Canada. Abstract entitled "The p55 and p75 TNF receptors both signal functional responses in normal rat mammary epithelial cells", Varela, L.M. and Ip, M.M., AACR Program, 36: 119, 1995.
2. Poster presented at Mammary Gland Gordon Conference in New London, New Hampshire. Abstract entitled "The role of TNF $\alpha$  in mammary gland development" Varela, L.M. and Ip, M.M. Poster selected for special presentation, for which a Gordon Conference travel award was received.

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## FIGURE LEGENDS

**Figure 1. Expression of TNF receptor mRNA in normal MEC.** Equal amounts of poly A<sup>+</sup> mRNA (~1.5 µg) from freshly isolated MEC (from 50-day old female rats) were loaded into each lane, and equal cpm ( $2 \times 10^6$ /ml) of <sup>32</sup>P-labeled p55 and p75 TNF receptor cDNA probes were used for hybridization. The p55 blot was exposed to film for 20 hours, and the p75 blot was exposed for 6 days. The data shows that both TNF receptors are expressed in MEC, with a single 2.3 kB species for the p55 receptor, and three transcripts of 5.7, 4.2, and 2.8 kB for the p75 receptor.

**Figure 2. Effect of TNF receptor agonistic antibodies on [<sup>3</sup>H]-thymidine incorporation.** Various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were tested for their ability to affect [<sup>3</sup>H]-thymidine incorporation by normal MEC in culture. This figure shows that EGF, TNFα and the 1:1000 dilution of the p55 receptor antibody were able to significantly stimulate thymidine incorporation (~3 fold), while the p75 receptor antibody had no effect. \*Significantly different than 0.1 ng/ml EGF control, P<0.05, n=3. This is representative of 3 separate experiments, each performed in triplicate.

**Figure 3. Effect of EGF, TNFα, or TNF receptor agonistic antibodies on MEC morphology.** Cells were cultured in suboptimal, serum-free medium until day 5; the medium was then changed and either EGF (10 ng/ml), TNFα (40 ng/ml), or various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were added and morphology was quantitated from 42-48 hours. This figure shows that organoids cultured in the 0.1ng/ml (low) EGF control medium were end bud in appearance, defined by their "simple" lobular structure and "rust" color, while treatment with either EGF, TNFα, or the 1:1000 dilution of either the p55 or p75 antibody was able to shift the morphology of the organoids toward the more differentiated lobulo-alveolar phenotype. Alveolar colonies are defined by their more complex lobular structure, darker gray or black color, and lipid droplets. The figure also shows that treatment significantly increased both the number and extent of ductal projections emanating from the colonies. Representative of three experiments.

**Figure 4. Effect of EGF, TNFα, or TNF receptor agonistic antibodies on end bud colony morphology.** MEC were cultured until day 5 in suboptimal medium; the medium was then changed and EGF (10 ng/ml), TNFα (40 ng/ml), or various dilutions of either the p55 or p75 TNF receptor agonistic antibodies were added. The morphological appearance of the MEC was quantitated from 42-48 hours of treatment. The graph shows that in the 0.1 ng/ml EGF control, the colonies are primarily (~85%) end buds, while in EGF- or TNFα- treated groups, the percentage of end bud-like colonies significantly decreased to ~20-25% of the total colonies. Interestingly, both TNF receptor agonistic antibodies were able to affect the morphology in a

concentration-dependent manner, with the 1:1000 dilution of each having the greatest effect. \*Significantly different than 0.1 ng/ml EGF control,  $P < 0.05$ ,  $n = 3$ . This figure is representative of three separate experiments.

**Figure 5. Effect of EGF,  $\text{TNF}\alpha$ , or TNF receptor agonistic antibodies on alveolar colony morphology.** MEC were cultured until day 5 in suboptimal medium; the medium was then changed and EGF (10 ng/ml),  $\text{TNF}\alpha$  (40 ng/ml), or various dilutions of either the p55 or p75 TNF receptor agonistic antibodies were added. The morphological appearance of the MEC was quantitated from 42-48 hours of treatment. The graph shows that in the 0.1 ng/ml EGF control, the colonies are only ~10% alveolar, while in EGF- or  $\text{TNF}\alpha$ - treated groups, the percentage of alveolar-like colonies significantly increased to ~75-80% of the total colonies. Interestingly, both TNF receptor agonistic antibodies were able to stimulate the morphological development of the MEC in a concentration-dependent manner, with the 1:1000 dilution of each having the greatest effect. \*Significantly different than 0.1 ng/ml EGF control,  $P < 0.05$ ,  $n = 3$ . This figure is representative of three experiments.

**Figure 6. Effect of EGF,  $\text{TNF}\alpha$ , or TNF receptor agonistic antibodies on casein accumulation by MEC in suboptimal (0.1 ng/ml EGF) medium.** On day 5, the medium was changed and either EGF (10 ng/ml),  $\text{TNF}\alpha$  (40 ng/ml), or various dilutions of the p55 or p75 TNF receptor agonistic antibodies were added and the cells were incubated for 48 hours. The MEC embedded with the EHS matrix were then harvested and equivalent amounts of protein (20  $\mu\text{g}/\text{lane}$ ) were subjected to Western blot analysis with anti-casein antiserum. This Western blot shows that EGF significantly increases the accumulation of all 4 forms of casein ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$ ) by MEC, while  $\text{TNF}\alpha$  had the opposite effect, with primarily the  $\alpha_2$ ,  $\beta$ , and  $\gamma$  forms being decreased. This figure also shows that the TNF receptor agonistic antibodies had opposing effects; MEC treated with the 1:1000 dilution of the p55 receptor antibody showed a decreased accumulation of  $\alpha_2$ ,  $\beta$ , and  $\gamma$  casein, but cells treated with the 1:10<sup>4</sup> and 1:10<sup>5</sup> dilution of the p75 antibody showed an increase in accumulation of primarily  $\beta$  and  $\gamma$  caseins. This figure is representative of three experiments.

**Figure 7. Effect of  $\text{TNF}\alpha$  or TNF receptor agonistic antibodies on casein accumulation by MEC in optimal (10 ng/ml EGF) medium.** On day 5, the medium was changed and either  $\text{TNF}\alpha$  (40 ng/ml) or various dilutions of the p55 or p75 TNF receptor agonistic antibodies were added, and the cells were incubated for 48 hours. The MEC embedded with the EHS matrix were then harvested and equivalent amounts of protein (8  $\mu\text{g}/\text{lane}$ ) were subjected to Western blot analysis with anti-casein antiserum. This Western blot shows that  $\text{TNF}\alpha$  significantly decreases the accumulation of all 4 forms of casein ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$ ) by MEC, with the primary effect on  $\alpha_2$ ,  $\beta$ , and  $\gamma$ . This figure also shows that the TNF receptor agonistic antibodies again had opposing effects; MEC treated with the 1:10<sup>3</sup> and 1:10<sup>4</sup> dilutions of the p55 receptor antibody showed a decreased accumulation of  $\alpha_2$ ,  $\beta$ , and  $\gamma$  casein, but cells treated with the 1:10<sup>4</sup> and 1:10<sup>5</sup> dilutions of the p75 antibody showed an increase in casein accumulation. This figure represents one experiment, performed in triplicate.

**Figure 8. Quantitation of casein accumulation by MEC in optimal (10 ng/ml EGF) medium.** The relative intensities of all 4 casein bands in the Western blot in Figure 7 were quantitated by densitometric analysis, and this figure shows graphical representations of those quantitations. This series of graphs demonstrates that 48 hour  $\text{TNF}\alpha$  treatment of cells cultured in optimal medium decreased the accumulation of all 4 forms of casein, with the most pronounced decreases in  $\alpha_2$ ,  $\beta$ , and  $\gamma$  caseins. The figure also shows that treatment with the p55 TNF receptor antibody decreased casein accumulation, but to a lesser extent than  $\text{TNF}\alpha$ , and these decreases were the most pronounced with the 1:10<sup>3</sup> and 1:10<sup>4</sup> dilutions. In addition, the graphs show that treatment with the 1:10<sup>4</sup> and 1:10<sup>5</sup> dilutions of the p75 TNF receptor antibody modestly increased the accumulation of all 4 forms of casein by the MEC.

**Figure 9. Effect of TNF receptor agonistic antibodies on casein synthesis.** MEC were cultured in optimal (10 ng/ml EGF) medium until day 5; the medium was then changed and various dilutions of either the p55 or p75 TNF receptor agonistic antibodies were added and the cells were incubated for 48 hours. Cells were labeled with [<sup>35</sup>S]-methionine in methionine-free medium for the last 24 of the 48 hours. The radiolabeled casein proteins were immunoprecipitated, electrophoresed, and subjected to fluorography, and the amounts of newly synthesized casein proteins were quantitated by densitometric analysis. This figure shows that the antibodies had opposing effects on casein synthesis by MEC. Treatment with the p55 TNF receptor antibody caused a suppression of casein synthesis, with the greatest decreases occurring in  $\alpha_1$  and  $\gamma$ . In contrast, treatment with the p75 TNF receptor antibody stimulated synthesis of  $\alpha_1$  and  $\gamma$  caseins, while  $\alpha_2$  and  $\beta$  were unaffected. This figure represents one experiment, performed in triplicate.

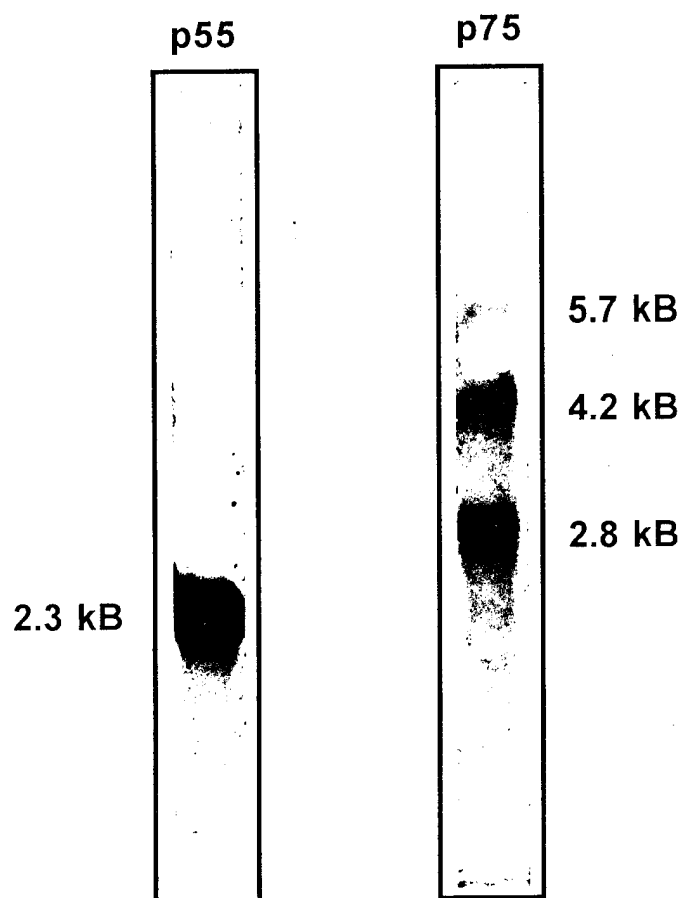


Figure 1

Figure 2

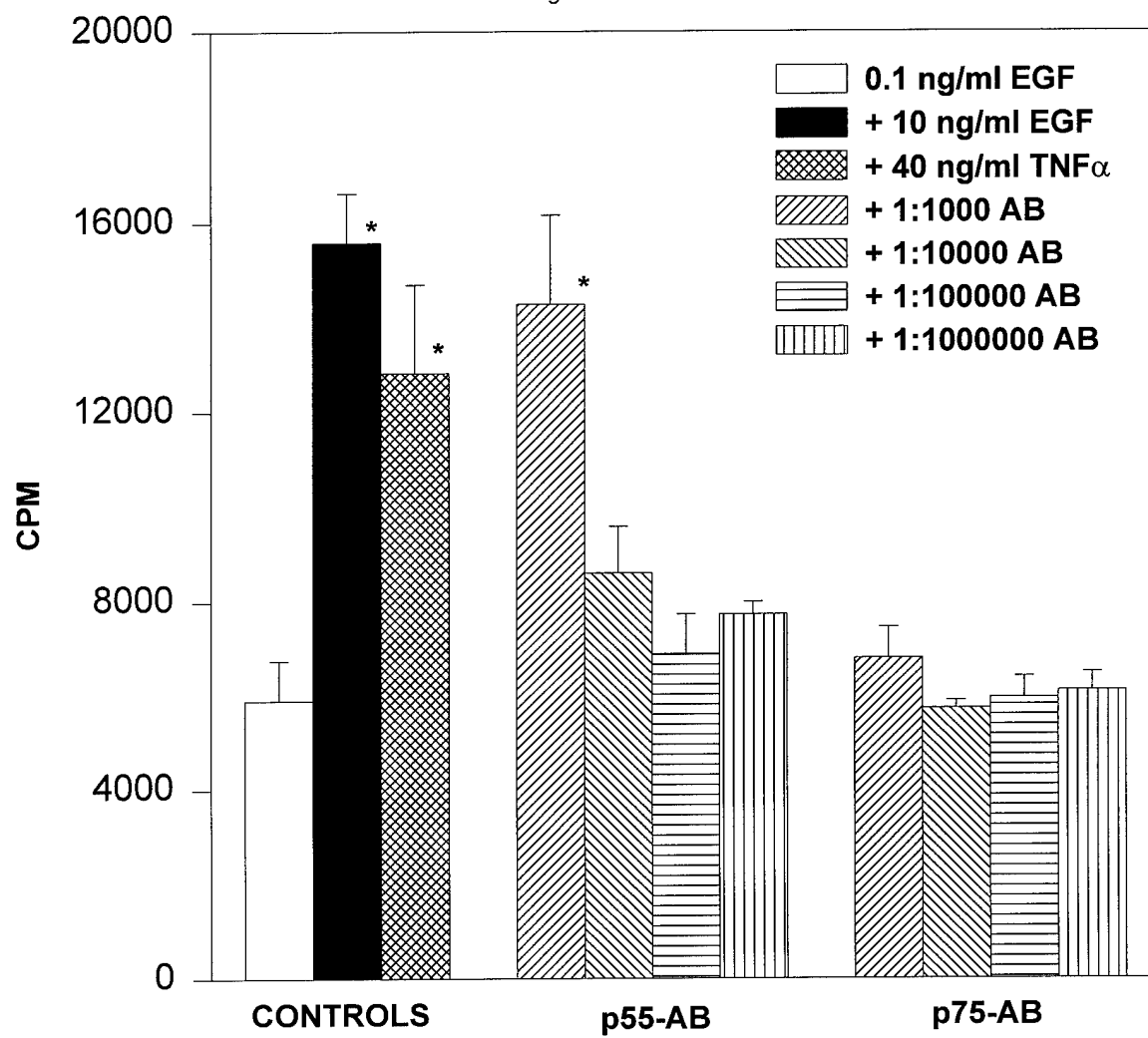
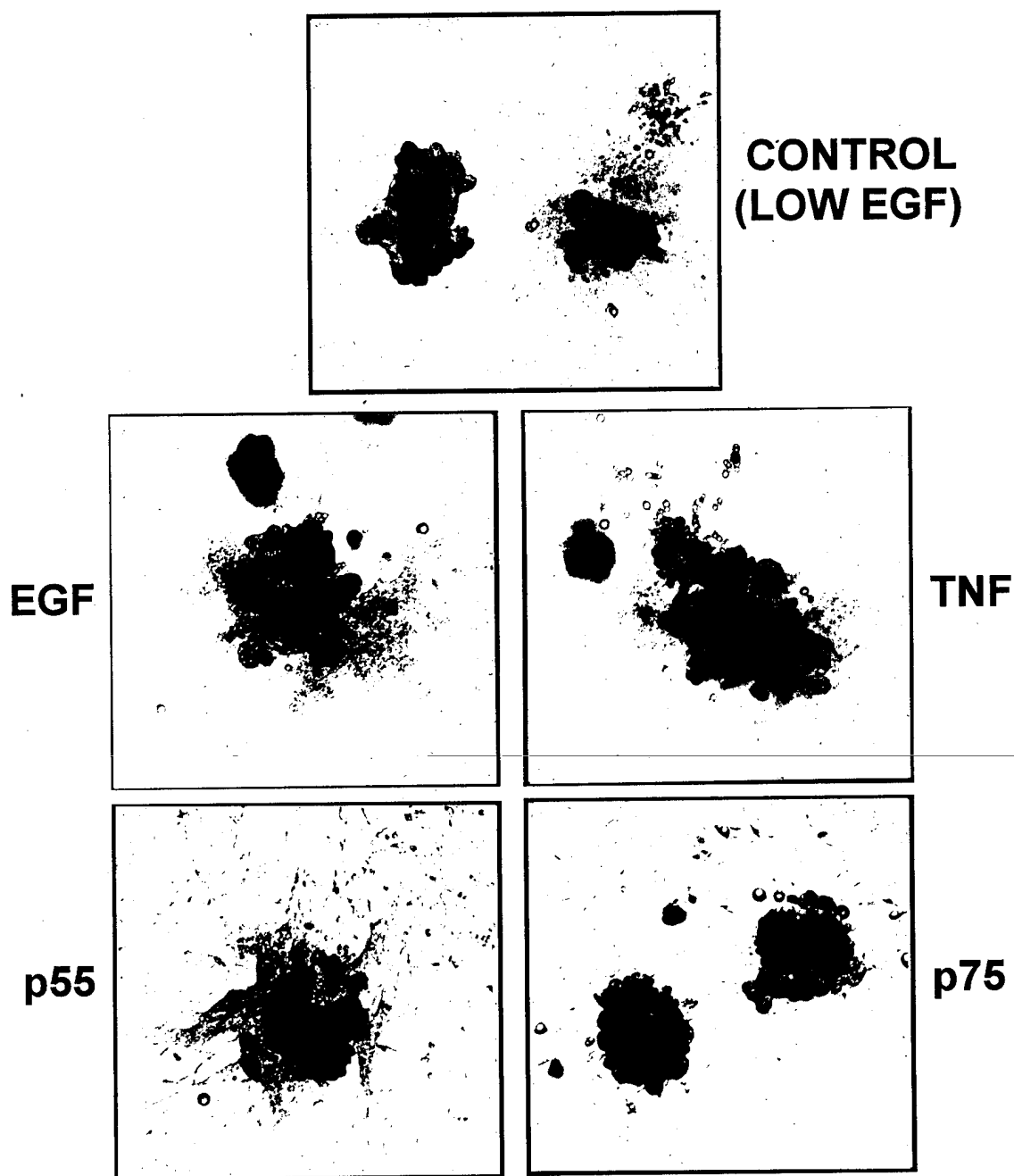


Figure 3



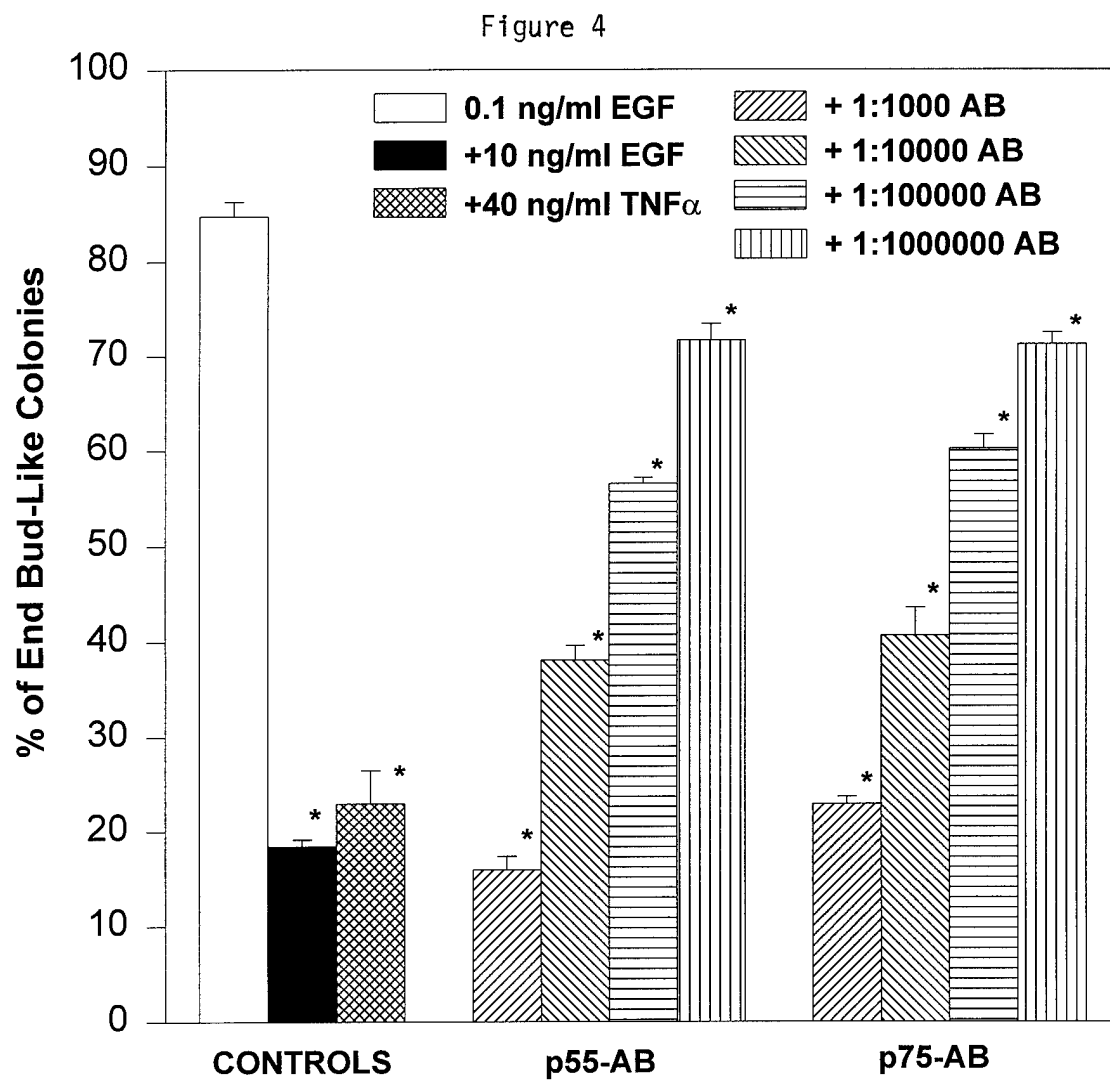
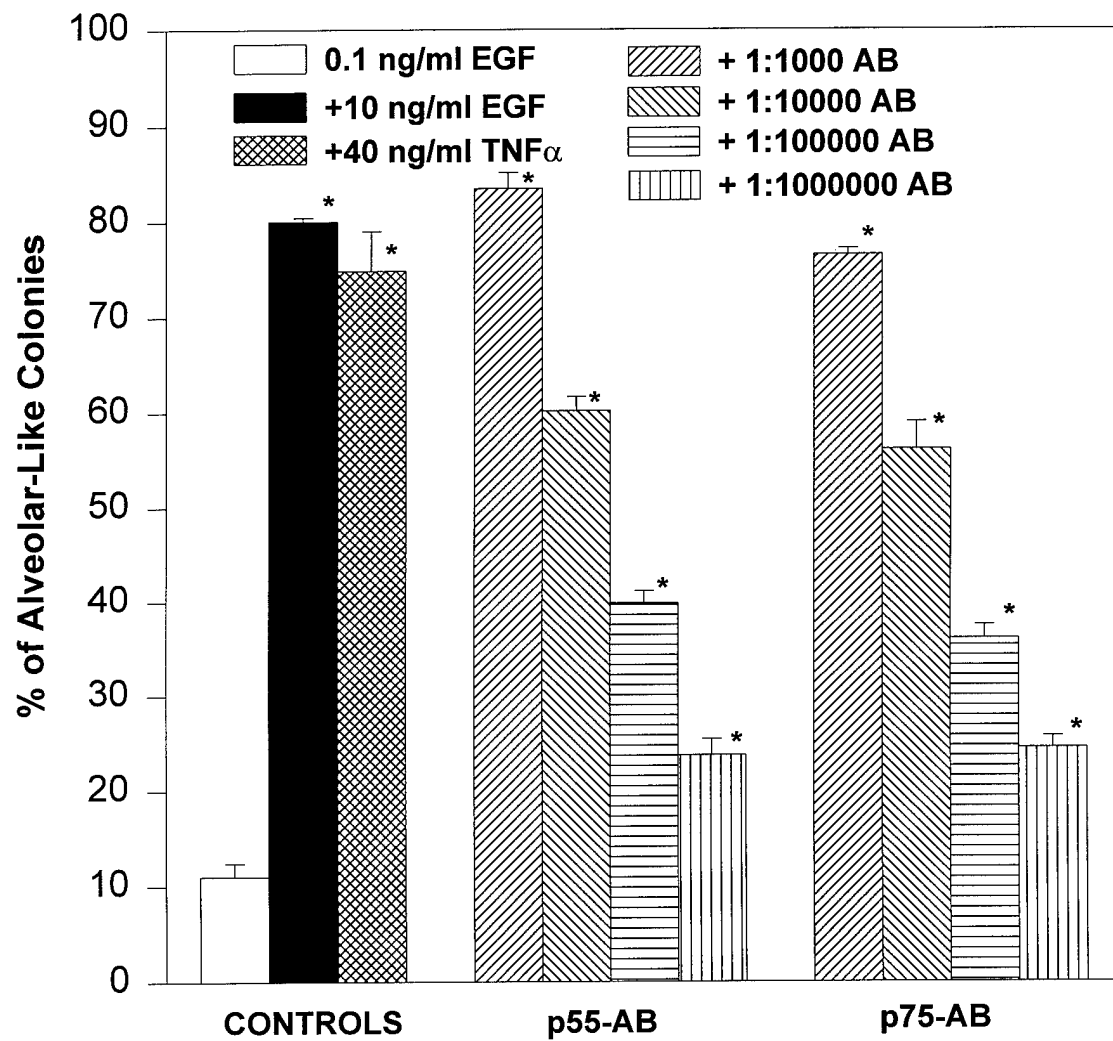


Figure 5





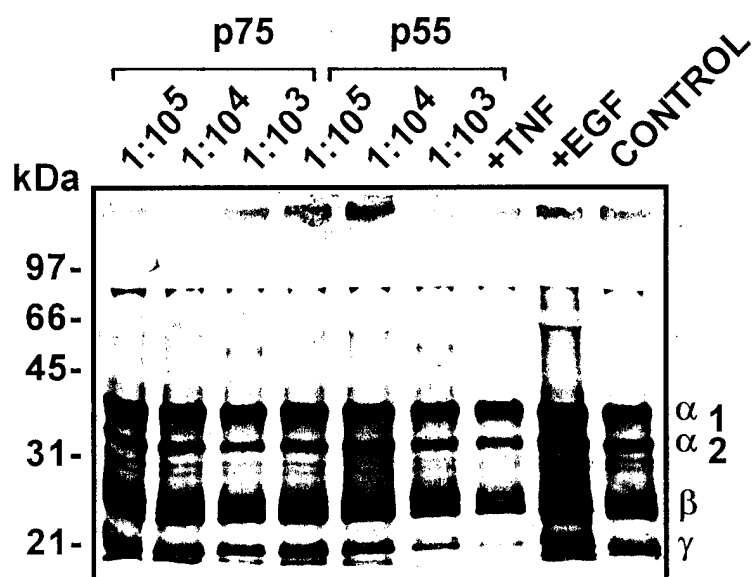


Figure 6

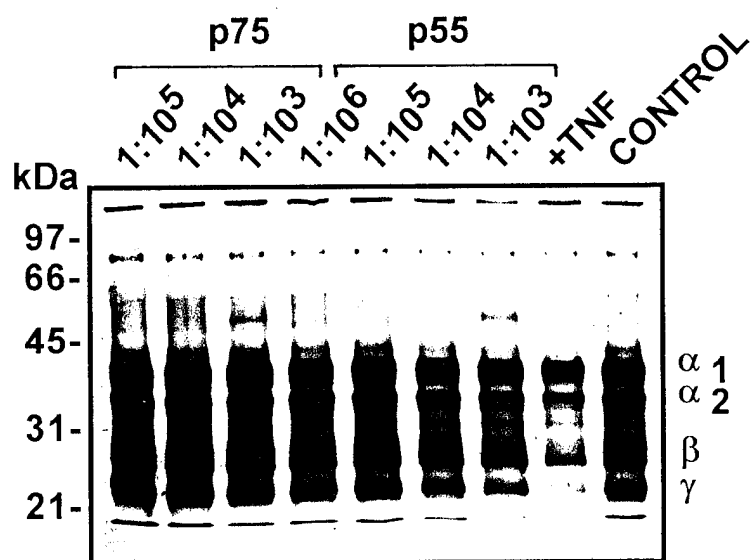


Figure 7

Figure 8

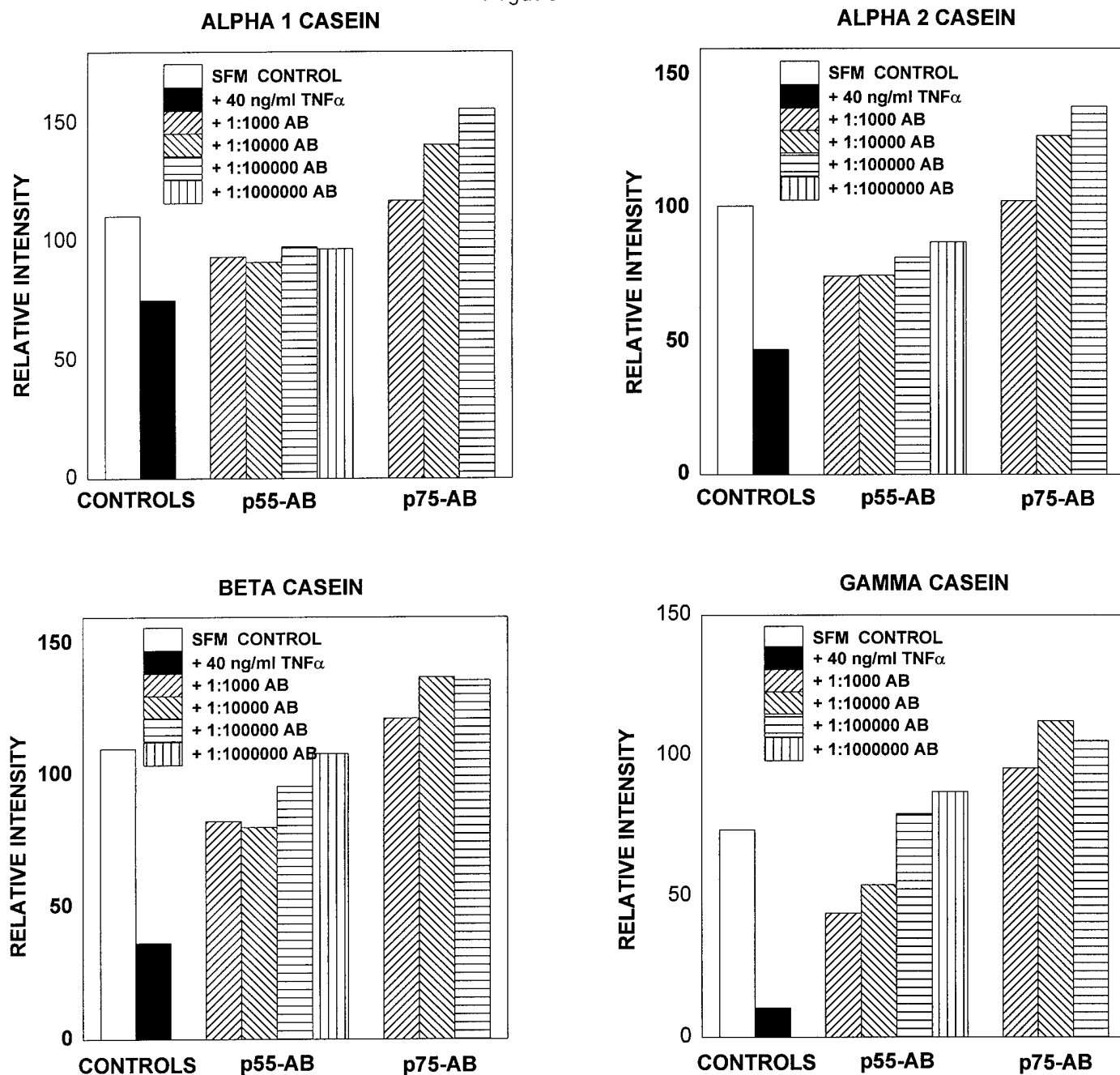


Figure 9

